行政院國家科學委員會專題研究計畫 成果報告

以突變種 TAT-RhoA 融合蛋白探討 RhoA 及其他訊息傳遞因子在調控血管平滑肌收縮的角色

計畫類別： 個別型計畫
計畫編號： NSC92-2320-B-006-032-
執行期間： 92 年 08 月 01 日至 93 年 07 月 31 日
執行單位： 國立成功大學解剖學科

計畫主持人： 江美治

報告類型： 完整報告

處理方式： 本計畫涉及專利或其他智慧財產權，1 年後可公開查詢

中華民國 93 年 11 月 03 日
行政院國家科學委員會補助專題研究計畫

成果報告 □ 期中進度報告

（計畫名稱）

計畫類別：□ 個別型計畫 □ 整合型計畫
計畫編號：NSC 92-2320-B-006-032
執行期間：2002年08月01日至2004年07月31日

計畫主持人：江 美 治
共同主持人：
計畫參與人員：

成果報告類型：□ 精簡報告 □ 完整報告

本成果報告包括以下應繳交之附件：
□ 赴國外出差或研習心得報告一份
□ 赴大陸地區出差或研習心得報告一份
□ 出席國際學術會議心得報告及發表之論文各一份
□ 國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□ 涉及專利或其他智慧財產權，□ 一年 □ 二年後可公開查詢

執行單位：國立成功大學
中華民國 年 月 日
ABSTRACT

Smooth muscle contractility is regulated by both \([\text{Ca}^{2+}]_i\) and \(\text{Ca}^{2+}\) sensitivity of the contractile apparatus. It has been suggested that phosphorylation at Thr696 of myosin light chain phosphatase (MLCP) regulatory subunit (MYPT1) and at Thr38 of the MLCP inhibitor protein CPI-17 results in inhibition of MLCP activity, thereby increasing \(\text{Ca}^{2+}\) sensitization. Experimental evidence concerning the roles of MYPT1 Thr696 in regulating smooth muscle contractility is highly controversial, however. This study was aimed to clarify whether phosphorylation of MYPT1 Thr696, MYPT1 Thr 850 or CPI-17 Thr38 mediates Rho kinase-regulated contraction and MLC\(_{20}\) phosphorylation in rat tail artery smooth muscle by examining the temporal changes of these parameters during agonist stimulation in the presence and absence of Rho-kinase inhibitor Y27632. Y27632 suppressed force activated by \(\alpha_1\)-adrenergic agonist phenylephrine (PE) or thromboxane A\(_2\) analogue U46619 with concomitant decreases in MLC\(_{20}\) phosphorylation. Immunoblotting using phospho-specific antibodies showed that PE (10 \(\mu\)M) and U46619 (0.3 \(\mu\)M) significantly enhanced the MYPT1 Thr850 phosphorylation while Thr696 phosphorylation was hardly increased. On the other hand, PE increased CPI-17 phosphorylation to higher extent than that of U46619. Y27632 inhibited both agonist-induced MYPT1 Thr850 phosphorylation to below resting levels without significant effects on CPI-17 phosphorylation. Furthermore, Y27632 dose-dependently inhibited force generation and \([\text{Ca}^{2+}]_i\), with force being suppressed to a greater extent than that of \([\text{Ca}^{2+}]_i\). These results suggest a temporal correlation between MYPT1 Thr850, MLC\(_{20}\) phosphorylation and contraction. PE may activate both Rho-kinase-MYPT1 Thr850 and PKC-CPI-17 signaling pathways to regulate contraction whereas U46619 mainly activates Rho-kinase-MYPT1 Thr850 pathway.

Keywords: Rho-kinase, \(\text{Ca}^{2+}\) sensitization, MLC\(_{20}\) phosphorylation, MYPT1, CPI-17, vascular smooth muscle contraction
INTRODUCTION

Cytoplasmic ionized Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is the primary regulator of smooth muscle contraction. A rise in [Ca\(^{2+}\)]\(_i\) activates Ca\(^{2+}\)/calmodulin-dependent myosin light chain kinase, which in turn phosphorylates 20 kDa regulatory myosin light chain (MLC\(_{20}\)), thereby triggering contraction. Experimental evidence has however, shown that [Ca\(^{2+}\)]\(_i\) levels are not always parallel with contractile force. Earlier studies showed that receptor agonists stimulate greater contraction than high K\(^+\)-depolarization does at similar [Ca\(^{2+}\)]\(_i\) (Bradley et al., 1987; Himpens et al., 1990). An increase in force and/or MLC\(_{20}\) phosphorylation at a constant [Ca\(^{2+}\)]\(_i\) is referred to as Ca\(^{2+}\) sensitization. It is now accepted that the mechanisms of receptor-mediated excitation-contraction coupling of smooth muscle include an important component attributing to Ca\(^{2+}\) sensitization (Somlyo et al., 1994). Ample documents have shown that G protein-coupled receptor activation increases the Ca\(^{2+}\) sensitivity of MLC phosphorylation and contraction in all types of smooth muscle through the inhibition of MLCP (Kitazawa et al., 1991; Kubota et al., 1992; Somlyo et al., 1994)

MLCP is a heterotrimeric enzyme with a 38 kDa type-1 phosphatase catalytic subunit isoform (PP1C), a 110-130 kDa myosin-targeting subunit (MYPT1) and a 20 kDa subunit of unknown function (Hartshorne et al., 1998). So far, two major pathways have been proposed for the regulation of MLCP. First, RhoA-associated kinase (Rho-kinase) phosphorylates Thr696 of MYPT1, which inhibits the phosphatase activity (Kimura et al., 1996); (Feng et al., 1999). Experimental evidence concerning the roles of MYPT1 Thr696 in regulating smooth muscle contractility is highly controversial, however (Ito et al., 2003; Kitazawa et al., 2003; Niiro et al., 2003; Seko et al., 2003). A second pathway involves an inhibitor protein for MLCP, called CPI-17. Phosphorylation of CPI-17 at Thr-38 by PKC converts this protein to a potent inhibitor of the MLCP holoenzyme (Eto et al., 1997; Senba et al., 1999). Stimulation of arterial smooth muscle with agonists, GTP\(\gamma\)S, or PKC activator evokes CPI-17 Thr38
phosphorylation, which parallels the Ca\textsuperscript{2+} sensitization of contraction (Kitazawa et al., 2000).

The kinetics of the changes in phosphorylation agrees with the contraction is not well documented, however. Using site- and phospho-specific MYPT1 Thr696, MYPT1 Thr850 and CPI-17 Thr38 antibodies, we examined whether receptor agonists induce phosphorylation of MYPT1 Thr696, MYPT1 Thr850 or CPI-17 Thr38 in parallel with MLC phosphorylation and contraction in vascular smooth muscles.

**MATERIALS AND METHODS**

_Materials:_ Phenylephrine hydrochloride was purchased from Sigma (St. Lious, MO, USA), (15S)-hydroxy-11\(\alpha\),9\(\alpha\)-(epoxymethano)prosta-5Z,13E-dienoic acid (U46619) was from Cayman (Kalamazzo, MI, USA). (+)-(R)-trans-4-(1-aminoethyl)-N-(4-Pyridyl) cyclohexane-carboxamide dihydrochloride, monohydrate (Y-27632) was kindly provided by A. Yoshimura, Welfide Corporation, Osaka, Japan. Aequorin was obtained from J. R. Blinks at the Friday Harbor Lab. (Friday Harbor, WA, USA). Rabbit polyclonal antibodies against phospho-MYPT-1 (Thr850 and Thr696), phospho-CPI-17 and CPI-17 were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-MYPT1 antibody M130a that recognize both dephosphorylated and phosphorylated forms was from Covance (Richmond, Calif). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Chemicon (Temecula, CA, USA). All other chemicals were of reagent grade.

_Tissue preparation and isometric contraction measurement._ This study was conducted in conformity with the procedures described in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, and the procedures performed were in accordance with institutional guidelines. Male Sprague-Dawley rats weighing 400 ~ 550 g were used in this study. After the animal was anesthetized with pentobarbital (60 mg/kg, i.p.), the tail artery
was removed and placed in oxygenated (95% O₂-5% CO₂) physiological saline solution (PSS, pH7.4) with the following composition (in mM): 120 NaCl, 5.9 KCl, 25 NaHCO₃, 1.2 NaH₂PO₄, 11.5 dextrose, 1.2 MgCl₂ and 2.5 CaCl₂. The vessel was dissected free of adipose and connective tissues, and then cut into helical strips (1~2 mm wide and 8~9 mm long). The endothelium was gently rubbed off with a moistened cotton swab. Muscle strips were placed in tissue bathes with one end held in a muscle holder and the other end connected to a force transducer. After being stretched to the length which allows for maximal force production and being equilibrated at 37°C for at least 1 hr, muscle strips were stimulated two times for 10 min with 51 mM KCl-PSS (PSS in which 51 mM NaCl has been stoichiometrically replaced by KCl) to produce stable and reproducible contractile responses.

Measurement of intracellular [Ca²⁺], [Ca²⁺], and isometric force were measured simultaneously with bioluminescent protein aequorin as the indicator. Aequorin was loaded into muscle strips by a reversible hyper-permeabilization method as described previously (Jiang et al., 1987). Briefly, muscle strips were incubated in a series of four solutions at 4°C as follows: 15 min in solution A (120 KCl, 2 MgCl₂, 20 TES, 5 ATP, and 10 EGTA); 120 min in solution B (120 KCl, 2 MgCl₂, 20 TES, 5 ATP, and 0.1 EGTA and 0.42 mg/ml aequorin); 30 min in solution C (120 KCl, 10 MgCl₂, 20 TES, 5 ATP, and 0.1 EGTA); and 120 min in solution D (120 NaCl, 5.9 KCl, 10 MgCl₂, 11.5 dextrose, and 25 NaHCO₃). The concentration of calcium in solution D was slowly raised to a final value of 2.5 mM. The subsequent aequorin experiment was conducted at 37°C in a light-tight apparatus which was modified from an original design of J. R. Blinks. Light emission of aequorin was detected by a photomultiplier tube (Thron EMI model 9635QA), and the anode current was recorded through an amplifier and stored in a data recorder (Panasonic VHS, modified by A.R. Vetter, model 420G). Aequorin light signal and isometric force were simultaneously recorded on a Gould TA2000 recorder. At the end of each experiment, the muscle cells were lysed with
distilled water containing 10 mM CaCl₂ to obtain the total aequorin luminescence (Lₘₐₓ).

[Ca²⁺]ᵢ was calculated from fractional luminescence (L/Lₘₐₓ) according to an in vitro calibration curve (Jiang et al., 1987).

**Smooth muscle stimulation and protein extraction:** Tail artery strips denuded of endothelium were mounted in tissue baths, after a 1-hr equilibration and initial challenge to 51 mM KCl, tissues were stimulated by PE (10 µM) or U46619 (0.3 µM). In another set of experiment, muscle strips were preincubated with Y27632 for 15 min before the addition of PE (10 µM) or U46619 (0.3 µM). Muscle strips were snap-frozen in liquid nitrogen pre-cooled freon at the desired time points following agonist stimulation and stored at −80 °C until use. Frozen strips were allowed to thaw in an acetone-dry ice slurry containing 10% TCA and 10 mM DTT, after three 20 min washed in acetone containing 10 mM DTT, the muscle strips were homogenized in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% Na-deoxycholate, 0.2% SDS, 1 mM PMSF, 1 µM leupeptin, 1 µM aprotinin, and 1 µM pepstatin) with glass-to-glass microhomogenizers. The homogenate was clarified by centrifugation at 15,000 g for 15 min at 4 °C. The supernatant was collected for Western blot assay as described below. Protein content was determined by using a bicinchoninic acid protein assay kit (Pierce).

**Western blot:** Equal amounts of protein were resolved under reducing conditions on 15% (for CPI-17) or 8% (for MYPT1) SDS-polyacrylamide gel and transferred electrically onto nitrocellulose membranes. Membranes were blocked for 1.5 h in a Tris-buffered saline (TBS) solution containing 3% nonfat dry milk at room temperature before an overnight incubation with phosphospecific anti-MYPT1 Thr696 or Thr850 antibody (1:1000) or anti-CPI-17 Thr38 antibody (1:500) at 4 °C. The blots were washed and incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5000) for 1.5 hat room temperature.
Immunoreactive bands were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences). Membranes were then stripped of bound antibodies by incubation in a solution containing 62.5 mM Tris-HCl (pH6.8), 2% SDS, and 10 mM DTT for 30 min at 50℃ with agitation. The blots were reprobed with anti-MYPT-1 (1:7500) or anti-CPI-17 (1:1,000) for a loading control.

Measurement of myosin light chain phosphorylation: Myosin light chain phosphorylation was measured by two dimensional gel electrophoresis as described previously (Jiang et al., 1994). Homogenates of RTA strips were sequentially analyzed by isoelectric focusing gel electrophoresis (Pharmalytes of 80% pH 4.5-5.4 and 20% pH 3-10, Amersham-Pharmacia) in the first dimension and SDS-PAGE in the second dimension. The gels were silver stained (Heukeshoven et al., 1988) and analyzed by densitometry. Data are expressed as the ratio of phosphorylated MLC over total (phosphorylated plus unphosphorylated) MLC.

Statistical Analysis: Data were expressed as means ± SEM. Statistical evaluation was conducted by Student’s t-test for paired data. For multiple comparisons, ANOVA followed by Newman-Kuels analysis was used. P-values of less than 0.05 were considered to have statistical significance.

RESULTS

Effects of Rho-kinase inhibitor Y27632 on PE- and U46619-induced contraction and MLC_{20} phosphorylation

As illustrated in Fig 1A, exposure of RTA smooth muscle to 10 μM PE caused an immediate contraction that attained a peak within 30 s and was sustained for up to 30 min of
observation. Preincubation with 2 µM Y27632 inhibited both initial and sustained phase of contraction to about 50%. In parallel to contraction, the phosphorylation of MLC$_{20}$ promptly increased after adding 10 µM PE and reached a peak at 1 min, thereafter, gradually declined to a level that still significantly higher than the resting level (Figure 1B). On the other hand, the thromboxane A$_2$ analogue U46619 provoked a slower contraction than that of PE. The contractile force reached its peak at 5 min and then maintained for the observation period of 30 min after 0.3 µM U46619 stimulation. Pretreatment with 1 µM Y27632 not only inhibited ~50% force but also slowed the rate of contraction (Figure 2A). The MLC20 phosphorylation increased to a maximum level at 2.5 min (23.4 ± 3.5%) and then decreased to 10.7 ± 2.4% at 30 min. However, 1 µM Y27632 has little effect on U46619-stimulated MLC$_{20}$ phosphorylation except that at 10 min (Figure 2B).

**Phosphorylation of MYPT1 at Thr696 and Thr850 in RTA strips**

Levels of MYPT1 phosphorylation at both Thr696 and Thr850 were examined. As shown in Figure 1D, stimulation with PE (10 µmol/L) resulted in a 2-fold increase in MYPT1 phosphorylation at Thr850 within 1 min. Thereafter, levels of phosphorylated MYPT1 gradually declined but remained elevated even after 30 min. Pretreatment with Rho-kinase inhibitor Y-27632 (2 µmol/L) completely abrogated PE-induced phosphorylation of MYPT1$_{\text{Thr850}}$. However, phosphorylation of MYPT1 at Thr696, a crucial inhibitory site for MLCP, was hardly elevated in response to PE stimulation. The pretreatment with 2 µM Y27632 also did not affect the phosphorylation levels of MYPT1$_{\text{Thr696}}$ (Figure 1C). Significant increase in MYPT1 Thr850 phosphorylation was also observed on stimulation with U-46619 (0.3 µmol/L). Again, the phosphorylation induced by U-46619 was suppressed to a level even lower than that at resting by 1 µM Y-27632 (Figure 2D). In addition, 0.3 µM U46619 stimulation cased a slow increase in MYPT1 phosphorylation at Thr696, which reached a peak at 10 min, and 1 µM Y27632 pretreatment abolished this effect (Figure 2C). Generally,
the phosphorylation extents of MYPT1 at both sites were more markedly enhanced by U46619 than by PE.

**Phosphorylation of CPI-17 in RTA strips**

Phosphorylation of endogenous CPI-17 in cultured rat aorta smooth muscle cells was measured by immunoblotting methods using anti-phosphoCPI-17 Thr38 antibody. Exposure of RTA smooth muscle to 10 μM PE caused an immediate phosphorylation of CPI-17 that attained a peak within 30 sec and then dramatically decreased to near resting level. PDBu, a PKC activator, potently enhanced CPI-17 phosphorylation, regardless of it produced a slower and small contraction than that of PE (data not shown). The phosphorylation of CPI-17 was not affected by 2 μM Y27632 (Figure 3A). Ro 31-8220, a PKC inhibitor, potently inhibited the peak phosphorylation level of CPI-17 to near basal level. Stimulation of RTA smooth muscle with 0.3 μM U46619 also increased CPI-17 phosphorylation which peaked at 30 sec (Figure 3B). In contrast to the results of MYPT1 phosphorylation, PE is a more effective stimulant than U46619 in activating CPI-17 phosphorylation.

**Effects of Rho-kinase inhibitor Y27632 on PE-induced changes in force and [Ca^{2+}] in intact RTA strips**

To explore the involvement of Rho-kinase in regulation of Ca^{2+} sensitization and mobilization, we examined the effects of Y27632 on force and [Ca^{2+}], measured simultaneously in response to PE stimulation. As summarized in Figure 4, 10 μM PE increased [Ca^{2+}], to 192.6 ± 14.5% of resting levels (194 ± 35 and 105 ± 21 nM for PE-stimulated and control, respectively). The addition of Y27632 (1~10 μM) dose-dependently decreased force and [Ca^{2+}]. Interestingly, 10 μM Y27632 resulted in significant decreases in force but not [Ca^{2+}]. At 3 μM Y27632, force was inhibited by more than 60% while [Ca^{2+}], was inhibited by ~15% only (p<0.05). As 10 μM Y27632 was added,
force was inhibited by 90% while [Ca$^{2+}$], was inhibited by ~23% only (p<0.05). Thus, contractile force was inhibited to a greater extent than that of [Ca$^{2+}$], indicating that Rho-kinase regulates contraction mainly through modulating Ca$^{2+}$ sensitivity and, to a smaller extent, Ca$^{2+}$ mobilization in tail artery smooth muscle.

**DISCUSSION**

In the present study, we found an uncoupling of PE/U46619-induced force generation and phosphorylation of MYPT1 at Thr695 in rat tail artery smooth muscle. Phosphorylation of MYPT1 Thr850 is responsible for PE/U46619-mediated Ca$^{2+}$ sensitization. Furthermore, PE may activate PKC-CPI-17 signaling pathway to regulate contraction in RTA.

Both agonists used in this study caused an initial and sustained contraction of RTA smooth muscle. U46619-induced a slower initial rate and a lower level of contraction than that of PE. The kinetic of MLC$_{20}$ phosphorylation was agreed with force development during PE stimulation. Preincubation with Y27632 suppressed both initial and sustained contraction and MLC$_{20}$ phosphorylation. In the presence of U46619 there is a sustained increase in force even after the phosphorylation of MLC$_{20}$ dramatically declines. We assume that force is maintained in this case by thin filament regulatory mechanisms that have previously been described to include a PKC-dependent ERK1/2 activation leading to phosphorylation of caldesmon, thereby, reversing the inhibitory effect of caldesmon on actin-activated actomyosin ATPase (Dessy *et al*., 1998).

It has previously been reported that MP activity is regulated by a signaling cascade that leads to the phosphorylation of MYPT1 on Thr695 (in the chicken 133 kDa MYPT1 sequence) by Rho-kinase and results in an inhibition of phosphatase activity (Feng *et al*. 1999). To our
surprised, the phosphorylation of MYPT1 at Thr696 is not stimulated during PE- or U46619-evoked contraction. In addition, Y27632 also exerted no effects on PE-stimulated MYPT1 Thr696 phosphorylation even 10 µM of Y27632 was added, which almost abolished the contraction (data not shown). In contrast, phosphorylation of MYPT1 at Thr850 was markedly increased by both agonists. Furthermore, Y-27632 effectively inhibited Thr850 phosphorylation and contraction activated by PE and U46619. Our results are consistent with previous reports showing that G-protein activators only stimulated MYPT1 phosphorylation at Thr850, not Thr696 (Kitazawa et al., 2003). These suggest that Rho-kinase is activated in response to G-protein activation and is responsible for the phosphorylation of Thr850, which does not have clear functional consequences (Feng et al. 1999). Interestingly, Velasco et al. have recently reported that phosphorylation of Thr850 reduces the affinity of N-terminal fragments of MYPT1 for myosin, however. The involvement of MYPT1 Thr850 phosphorylation in smooth muscle Ca\(^{2+}\) sensitization is under investigation now.

CPI-17, a phosphorylation-dependent inhibitory protein of myosin phosphatase, has been suggested to be the downstream effector of the PKC that may be responsible for the increased contractility (Kitazawa et al., 1999; Woodsome et al., 2001), as PKC phosphorylates and activates CPI-17. It has also been reported that other kinase such as Rho-kinase can phosphorylate CPI-17 (Koyama et al., 2000). Thus, we tested the effect of Rho-kinase inhibitor Y27632 on both agonist-provoked CPI-17 phosphorylation. Exposure of RTA to PE caused a rapid increase in CPI-17 phosphorylation within 1 min, paralleled that of initial contraction. Pretreatment with Y27632 has no effect on this response, while Ro 31-8220 potently inhibited CPI-17 Thr38 phosphorylation. These results imply that PE activated PKC, not Rho-kinase, to enhance CPI-17 phosphorylation in RTA. On the other hand, U46619 caused only moderate increase in CPI-17 phosphorylation. In contrast to our result, Kitazawa et al. have reported the involvement of RhoA/ROCK in CPI-17 phosphorylation in response
to histamine stimulation. These conflicting results may be due to the difference in tissues or stimulants.

Result from the present study showed that Rho-kinase inhibitor Y27632 markedly inhibited PE-activated force with only small decreases in [Ca$^{2+}$], provide evidence to support the notion that Rho/Rho-kinase modulate calcium sensitivity of smooth muscle contraction. Because U46619 did not consistently induce significant [Ca$^{2+}$], increases in rat tail artery preparations, the effects of Y27632 were not examined.

In summary, the present study demonstrated a temporal correlation between MYPT1, MLC$_{20}$ phosphorylation and contraction. The phosphorylation of MYPT1 at Thr696 was not regulated during contraction and may not depend on Rho-kinase. PE may activate Rho-kinase-MYPT1 Thr850 and PKC-CPI-17 signaling pathway to regulate contraction, while U46619 may mainly through activating Rho-kinase-MYPT1 Thr850 pathway.

**FIGURE LEGENDS**

**Figure 1.** Changes in force, MLC$_{20}$ phosphorylation and MYPT1 phosphorylation induced by 10 µM PE in the rat tail artery strip. A: PE was added for various intervals. Contraction consisted of an initial peak followed by a sustained contraction. Y27632 (2 µM) was applied 15 min before the application of PE. B: Summary of the time course of changes in MLC$_{20}$ phosphorylation induced by PE in the absence (control) and the presence of 2 µM Y27632. C: Top: representative Western blots showing the effect of pretreatment with 2 µM Y27632 on MYPT1 Thr696 phosphorylation at the indicated time after stimulation with PE. Middle: representative Western blots of total MYPT1. Bottom: summary of densitometric
results. Results were normalized against time 0 and represented the mean of three independent experiments. D: Top: representative Western blots showing the effect of pretreatment with 2 µM Y27632 on MYPT1 Thr850 phosphorylation at the indicated time after stimulation with PE. Middle: representative Western blots of total MYPT1. Bottom: summary of densitometric results. Results were normalized against time 0 and represented the mean of three independent experiments. Data are presented as mean ± S.E.M.

Figure 2. Changes in force, MLC\textsubscript{20} phosphorylation and MYPT1 phosphorylation induced by 0.3 µM U46619 in the rat tail artery strip. A: U46619 was added for various intervals. Contraction slowly increased to a sustained phase. Y27632 (1 µM) was applied 15 min before the application of U46619. B: Summary of the time course of changes in MLC\textsubscript{20} phosphorylation induced by U46619 in the absence (control) and the presence of 1 µM Y27632. C: Top: representative Western blots showing the effect of pretreatment with 1 µM Y27632 on MYPT1 Thr696 phosphorylation at the indicated time after stimulation with U46619. Middle: representative Western blots of total MYPT1. Bottom: summary of densitometric results. Results were normalized against time 0 and represented the mean of three independent experiments. D: Top: representative Western blots showing the effect of pretreatment with 1 µM Y27632 on MYPT1 Thr850 phosphorylation at the indicated time after stimulation with U46619. Middle: representative Western blots of total MYPT1. Bottom: summary of densitometric results. Results were normalized against time 0 and represented the mean of three independent experiments. Data are presented as mean ± S.E.M.

Figure 3. Effect of pretreatment with Y27632 on the phosphorylation of CPI-17 in rat tail artery. A: Top: representative Western blots showing the effect of pretreatment with 2 µM Y27632 on CPI-17 Thr38 phosphorylation at the indicated time after stimulation with PE. Middle: representative Western blots of total CPI-17. Bottom: summary of densitometric
results. B: Top: representative Western blots showing the time course of U46619 on CPI-17 Thr38 phosphorylation. Middle: representative Western blots of total CPI-17. Bottom: summary of densitometric results. Data are presented as mean ± S.E.M. of three independent experiments.

**Figure 4.** Effects of Y27632 on PE-activated force and \([\text{Ca}^{2+}]_i\). Aequorin-loaded RTA strips were stimulated with 10^{-5} M PE for 10~15 min before cumulative doses of Y27632 were added during the simultaneous recording of force and aequorin light levels. Summary of the Y27632 effects on PE-activated force and \([\text{Ca}^{2+}]_i\) in intact RTA smooth muscle. Force obtained before inhibitor treatment was defined as 100% and \([\text{Ca}^{2+}]_i\) was normalized to resting levels \((\text{[Ca}^{2+}]_r)\). Data were expressed as mean ± SEM of 5 independent experiments. *p<0.05 vs. value obtained before inhibitor was added.

**REFERENCES**


FENG, J., ITO, M., ICHIKAWA, K., ISAKA, N., NISHIKAWA, M., HARTSHORNE, D.J. & NAKANO,


Expression of CPI-17 and myosin phosphatase correlates with Ca(2+) sensitivity of protein kinase C-induced contraction in rabbit smooth muscle. J Physiol, 535, 553-64.
Figure 1
A.

| -Y27632: 0 0.5 1 2.5 5 10 30 (min) |
|------------------|------------------|
| p-CPI-17         | CPI-17           |
| -Y27632:         | -Y27632:         |
| p-CPI-17         | CPI-17           |

B.

| -Y27632 0 0.5 1 2.5 5 10 30 (min) |
|------------------|------------------|
| p-CPI-17         | CPI-17           |

Figure 3
Figure 4